Single-particle cryoEM reconstructions: Meeting the challenge

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Tremendous progress has been made in recent years in the acquisition and treatment of electron micrographs of biological samples. These developments now allow retrieving much higher-resolution information from the scrutinized object than was possible until recently. In the case of highly symmetric particles, this approach is providing electron density maps comparable to those obtained by X-ray crystallography at least for large assemblies of equivalent sizes. Such accomplishments were made recently with icosahedral viruses, a bacteriophage (1) and an insect virus (2), and now, as reported in this issue of PNAS (3), this approach has revealed the details of the triple-layered architecture of the medically important rotavirus. This work follows from the recent 3D reconstruction of the double-layered rotavirus subparticles at the same level of resolution (4). There has indeed been steady methodological progress in this field. The first 3D fold of a structural protein of a human virus determined by EM, the hepatitis B virus (HBV) core protein, was reported ≈12 years ago (5, 6). The EM map of the HBV core particles obtained at the time was of sufficient quality to resolve the secondary structure elements of an α-helix-rich HBV core protein. From these pioneering studies, we have now moved to an era in which the quality of the EM maps permits the tracing of entire polypeptide chains for much larger proteins. Although the reported high-resolution 3D reconstructions are still special cases because of their high symmetry, the advances are not necessarily limited to symmetrical particles, but are likely to apply to any rigid macromolecular assembly, provided that enough high-quality images are available to reconstruct the object.

Together with similarly impressive developments in cryo-electron tomographic methods, which allow the study of objects that are not rigid (like organelles or other complexes that are heterogeneous in shape or/and size) the whole field of structural biology is making a considerable leap forward, moving beyond "molecular" to reach the realm of cellular structural biology (reviewed in ref. 7).

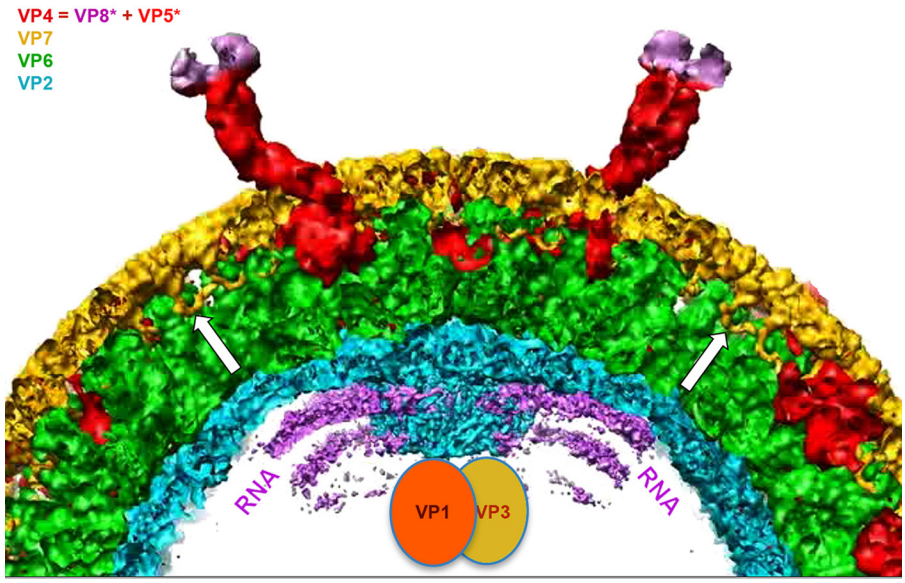

Fig. 1. Cross-section of a rotavirus particle, based on a surface rendering of a moderate-resolution cryoEM reconstruction of the intact virion (courtesy of Chen et al., ref. 3). The various protein components are colored as indicated by the labels in the upper left. VP1 and VP3, the polymerase and mRNA capping enzyme, respectively, are tethered to an inward-projecting part of the inner-shell protein, VP2. White arrows show where the N-terminal arms of the outer-layer protein, VP7, clamp onto the underlying VP6.

Rotaviruses are the most important viral agents of life-threatening gastroenteritis in children worldwide; their heavy disease toll has sparked efforts to develop protective vaccines, and today 2 live-attenuated vaccines have been licensed in many countries (8). The virus genome is composed of 11 segments of dsRNA, coding for 12 viral proteins. In addition to being important research targets for retrieving key information to combat the pathogen, the rotavirus particles are fascinating nano-objects of study for understanding macromolecular architecture in relation to biological function in general. The particle has a complex structure composed of concentric protein layers, as illustrated in Fig. 1. The polymerase and other replication enzymes are maintained within the particle, together with the genomic RNA. The proteins of the outer layer, VP7 and VP4, are used for cell entry, in a process in which they dissociate from the particle to allow translocation of a subviral, double-layered particle (DLP) across the membrane into the cytoplasm of the target cell. The DLP is composed of proteins VP6 (middle layer) and VP2 (inner layer) and an enzyme complex that includes polymerase and capping enzymes bound to the genomic RNA (illustrated in Fig. 1).

The process of membrane penetration is not understood at present, but a reported major conformational change in VP4 appears to be an important driving force (9). The DLPs become transcriptionally active in the cytoplasm, synthesizing capped mRNAs that are extruded through pores located at the 12 icosahedral vertices of the particles (10). Virus replication takes place in a specialized, nonmembrane-bound cytoplasmic compartment induced by the virus in the infected cell, called "viroplasm" (for a review of the rotavirus replication cycle, see ref. 11). The rotavirus assembly and exit pathway is complex, including budding of newly formed DLPs into the lumen of the ER, transiently acquiring a lipid envelope. This process involves interactions between the DLPs, assembled

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in the viroplasm, with viral protein NSP4, which is anchored in the ER membrane and serves as a receptor for the DLP. NSP4 becomes incorporated into the transient envelope during this process.

It is not known at what stage of assembly VP4 is recruited into the particle, but the structure suggests that it has to be added before VP7, which locks it in place. Protein VP7 eventually replaces the envelope through an unknown mechanism to make the triple-layered particles. The exact exit pathway of the virions and the timing of these events are not known. A final proteolytic maturation step takes place to render the new particles infectious, in which the VP4 spikes are cleaved into 2 components, VP8* (purple in Fig. 1) and VP5* (red in Fig. 1). This cleavage is required for the necessary conformational changes of the spike to allow virus penetration into target cells.

The important lesson from the rotavirus work is the wealth of information that can be obtained by pursuing complementary approaches, in this case X-ray crystallography and EM. Indeed, from the time when it was only possible to fit a rigid atomic model (the crystal structure of an isolated capsid component, for instance) into a cryoEM map to generate a rough model for the assembly, we have moved to having the possibility of observing subtle conformational changes of these molecules in the final assembly. These rearrangements result from their mutual interactions to form the particle and often provide important functional insight. For instance, in a parallel study, Aoki et al. (12) show that crystals of the isolated VP7 trimer in complex with a Fab fragment of a neutralizing antibody reveal a conformation of the VP7 trimer that is different from that in the virion. The intersubunit contacts are conserved, but there is a change in the relative orientations of the 2 domains composing the subunit. This change appears to be caused by the interaction with VP6. Furthermore, the N- and C-terminal ends of VP7 were disordered in the crystal, but in the particle the 3D reconstruction shows how the 3 N-terminal arms of a VP7 trimer grip the VP6 trimer that lies underneath (arrows in Fig. 1), adjusting to the overall shape of VP6 (3). In addition, the EM reconstruction shows that there are contacts between VP7 trimers in the surface lattice that are mediated by its N- and C-terminal arms, creating continuity across the VP7 layer and perhaps introducing cooperativity for disassembly.

Assembly and disassembly of the triple-layered rotavirus particle is controlled by calcium concentration.

We have thus moved from a time when EM provided the overall organization of a particle but not the detailed interactions to one where EM is providing the details. The comparison with X-ray crystallographic data on the individual components is providing a dynamic view of the molecule, showing the location of hinge regions and what changes occur on the folded molecule upon assembly into the particle.

An important further example is illustrated by the rotavirus calcium sensor. The whole process of assembly and disassembly of the triple-layered rotavirus particle is controlled by calcium concentration, the virus using the difference in calcium levels inside and outside a cell to its own advantage. Indeed, triple-layered particles are stabilized in the extracellular environment, but become unstable in the intracellular milieu where the calcium concentration is low. This is used for uncoating and entry, with disassembly of the VP7 layer as a result. The location of 2 Ca^{2+} binding sites in VP7 at the trimer interface explains why the metal is important for trimer stability, and the structure shows that it is the trimer that adopts the required shape to bind to the particle. Trimer dissociation thus entails disassembly of the VP7 layer from the virus surface, allowing the subsequent conformational changes in the spike for membrane disruption.

The EM structure also confirms previous observations made at lower resolution, that the presence of VP7 reorients the subset of VP6 trimers that directly surround a 5-fold vertex of the particle, which was postulated as the mechanism for transcription inhibition by VP7 (13). Indeed, the new structure reveals that this reorientation of VP6 transmits a signal to the inner layer, inducing a small conformational change in VP2, in the domain located adjacent to the 5-fold axes, which closes the transcript gate. The rotavirus calcium sensor during cell entry thus works via a cascade of events: destabilization of the VP7 trimer causes its dissociation from the particle, which induces a reorientation of the VP6 trimers around the 5-folds, which results in the release of a constraint on the VP2 domain just around the 5-fold axes, such that the gate opens.

The rotavirus cycle involves a journey through the cell for which many questions remain unanswered. The mechanism of membrane disruption is not understood, nor are the enveloping–de-enveloping steps during assembly. The structure of immature, enveloped particles has not been analyzed. The recent progress in EM, in combination with high-resolution light microscopy (14) and the available panoply of biophysical techniques that together constitute the leading edge of structural biology, are likely to provide many more important breakthroughs in understanding these processes. This information can in turn be used to identify ways to interfere with the virus cycle as curative treatment. Although rotavirus is but one example, this approach can be extended to other pathogens. Just as EM methodologies are crucial for studying cell biology, they are becoming a critical tool for gathering sufficient knowledge to effectively fight infectious diseases.

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